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(54) Title: IN VITRO ANTIBODY AFFINITY MATURATION USING ALANINE SCANNING MUTAGENESIS

(57) Abstract

A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided.

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TITLE OF THE INVENTION

IN VITRO ANTIBODY AFFINITY MATURATION USING ALANINE SCANNING MUTAGENESIS

CROSS-RELATED TO OTHER APPLICATIONS

This is a continuation of U.S. Serial No. 08/206,076 filed March 4, 1994, now pending.

BRIEF DESCRIPTION OF INVENTION

A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided. The method of the invention is a systematic means to achieve *in vitro* antibody maturation and uses alanine scanning mutagenesis. The invention is particularly exemplified with a set of single chain Fv (scFv) antibodies obtained by this technique. The resulting antibodies are directed against the V3 loop of HIV gp120, and show altered off-rates against the antigen compared to the starting antibody. Of particular interest are the altered antibodies which show improved (slower) off-rates to the antigen. Observed improvements have been as high as eleven-fold over wild-type.

SUMMARY OF THE INVENTION

A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Alanine-Scanning Mutagenesis. Each of the 27 amino acids in VH CDR3 of scFv P5Q was converted to alanine by site-directed mutagenesis. *E. coli* clones were induced to express scFv with IPTG. Single chain Fv, which is targeted to the periplasmic space by the fd phage gene3 signal sequence, was

extracted with EDTA. Periplasmic extracts were analyzed by BIAcore™, which measures antibody-antigen affinity by surface plasmon resonance (Fägerstam, 1991), and off-rates determined against an HIV gp120 V3 loop peptide. Results of the alanine scan, relative to P5Q, fall into four classes: i) slower off-rate, ii) faster off-rate, iii) no binding, and iv) minor or no change in off-rate. Standard deviation is ± 25%.

Figure 2. Amino Acid Randomization: Position 107. Arginine at position 107 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

Figure 3. Amino Acid Randomization: Position 111. Glutamic acid at position 111 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

Figure 4. Amino Acid Randomization: Position 112. Aspartic acid at position 112 was mutated to all amino acids by site-directed mutagenesis. Single chain Fv extracts were analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

Figure 5. Additive Effect of Combining Optimized Residues. A double mutant, containing the optimized residues, was constructed and analyzed by BIAcore. Percent change in off-rates is shown relative to P5Q.

Figure 6. Nucleotide and amino acid sequences of scFv P5Q with c-myc tail.

DETAILED DESCRIPTION OF THE INVENTION

The gp120 V3 domain of human immunodeficiency virus-1 (HIV-1) is a disulfide-linked closed loop of approximately 30 amino acids. The loop, in either native or synthetic form, binds to and elicits anti-HIV-1 antibodies.

The present invention relates to modified antibodies and methods of making modified. The invention is exemplified with modified HIV-1 immunoglobulins and methods of making these

modified HIV-1 immunoglobulins. The modified immunoglobulins of the present invention contain an altered complementary determining region 3 (CDR3) of HIV-1 neutralizing antibody.

The present invention also comprises a method of treating of preventing infection through the administration of a modified antibody to a suitable host. In one embodiment of the invention, the treatment or prevention of HIV infection through the administration of the modified HIV-1 immunoglobulin is described.

The present invention also comprises diagnostic kits useful for the detection or characterization of an antigen. Reagents for the kits may include DNA molecules encoding the modified antibodies or the modified antibodies or combinations thereof.

A method of mutagenizing antibodies to produce modified antibodies, modified antibodies, DNA encoding the modified antibodies as well as diagnostic kits and pharmaceutical compositions comprising the antibodies or DNA are provided. The method of the invention is a systematic means to achieve *in vitro* antibody maturation and uses alanine scanning mutagenesis. The invention is particularly exemplified with a set of single chain Fv (scFv) antibodies obtained by this technique. The resulting antibodies are directed against the V3 loop of HIV gp120, and show altered off-rates against the antigen compared to the starting antibody. Of particular interest are the altered antibodies which show improved (slower) off-rates to the antigen. Observed improvements have been as high as eleven-fold over wild-type.

Maturation was achieved through an alanine scan of complementary determining region 3 (CDR3) to identify positions critical to antigen binding. Critical positions were then randomized to identify amino acids that provided the slowest off-rates. Finally, clones were optimized through the combining of mutations.

The underlying principle of the method is the physical and chemical neutrality of alanine. Alanine is substituted throughout a stretch of amino acids, and its effects on binding (such as off-rate and on-rate) are evaluated using conventional methods. The number

of positions likely to be identified in this manner is relatively small. Once identified, these key positions may be randomized to all amino acids to identify the best amino acid solution at the position. Because all manipulations and evaluations are conducted *in vitro*, physiological bias is limited.

Present methods of *in vitro* antibody maturation are essentially random procedures in which the researcher generates clones with amino acid substitutions and evaluates them. The problem is that the number of substitutions necessary for a thorough evaluation is extremely large. For example, if one were to evaluate all random substitutions in CDR3, a region typically twenty-five residues in length, one would have to examine $9 \cdot 10^{27}$ possibilities. This is beyond the capabilities of present technologies.

Alanine scanning maturation enables the rapid identification of residues most likely to be important in binding. Using the example of a twenty-five residue stretch cited above, only twenty-five substitutions would be necessary. From this initial screen, amino acid positions likely to be critical to binding may be identified. The critical residues may then be randomized to identify the amino acids that optimize binding. Using this method, scFv antibodies with dissociation rates greater than ten-fold slower than the original scFv have been created.

Previous work in *in vitro* antibody maturation used one of two general approaches. In one approach, PCR recombination is used to substitute all or part of the VH and VL genes into libraries of scFv clones. In the second approach, random mutations are made throughout a CDR region of a scFv clone by the use of degenerate oligonucleotides. In both cases, clones were expressed as a phage fd gene 3 fusion surface protein. Higher affinity clones were identified using a panning assay followed by clonal purification of the phage.

Each approach has drawbacks. The PCR method is cumbersome, limited to the sequences of the B cell population, is essentially random in nature, and may introduce unwanted mutations through the PCR recombination step. The randomization approach

produces only a small fraction of the possible CDR changes. Neither approach allows immediate determination of changes in binding affinity because it is necessary to first generate an enriched population of suitable clones through panning. Both approaches detect only changes which result in improved binding; they do not identify positions for which the change weakened the binding. The latter class of change may include critical binding residues in which the appropriate amino acid solutions leads to improvement.

The method disclosed herein is systematic, thorough and unlikely to introduce unexpected or undesired mutations. All manipulations are done *in vitro*, which minimizes bias due to selection steps. Evaluation of clones is quantitative. In some cases, a key amino acid position may display poorer binding with alanine, but subsequent randomization may yield an amino acid solution which enables improved binding. Such mutations would not be detected by previous methods. Because the method of the present invention does not require phage expression for panning, the method can be used on scFVs, Fabs, and full length antibodies. Use is not restricted to a scFv for phage expression. Using the approach of the present invention, an anti-HIV V3 loop antibody was improved approximately eleven-fold.

Alanine scanning maturation of antibodies is a general method which may be used to improve binding of antibodies to their cognate antigens. The method has been used to identify critical residues in the scFv 447 which can be introduced into MAb447. Such changes may lead to significant improvement of the binding affinity of MAb447 against multiple species of HIV gp120 isolates. This improvement may increase the neutralization capability of the antibody, and significantly lower the effective dose.

Although the method and antibodies of the present invention are exemplified with scFv antibodies, it is readily apparent to those skilled in the art that the method may be used with other types of antibodies or with antibodies targetted against different

epitopes or antigens. Other types of antibodies include but are not limited to fragments of antibodies and full-length antibodies.

The molecular biology and immunological techniques of the present invention can be performed by standard techniques well-known in the art. See, for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides.

The cloned DNA molecules obtained may be expressed by cloning the gene encoding the altered antibody into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant modified antibodies. Techniques for such manipulations are well-known in the art.

In order to simplify the following Examples and the Detailed Description, certain terms will be defined.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Expression vectors include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Specifically designed vectors allow the shuttling of DNA between hosts, such as bacteria-yeast or bacteria-animal cells.

DNA encoding antibodies may also be cloned into an expression vector for expression in a host cell. Host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian and insect cells and cell lines.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to

transformation, transfection, protoplast fusion, and electroporation.

Expression of cloned DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with micro-injection into frog oocytes being preferred.

It is also well-known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variant.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of these examples.

EXAMPLE 1

Construction of mutations

Plasmid pP5Q was the starting vector for all mutagenic studies. Plasmid pP5Q is a derivative of p5H7 (Cambridge Antibodies). Plasmid pP5Q contains the VH and VL regions originally derived from MAb 447 (Gorney *et al.*) cloned as a single chain fragment variable (scFv).

Table 1 lists some of the oligonucleotide primers used for site-directed mutagenesis of complementary determining region 3 (CDR3) of MAb447. Primers were synthesized on either a model 381A DNA Synthesizer (Applied Biosystems, Foster City, CA) or a Cyclone™ Plus DNA Synthesizer (MilliGen/Biosearch, Marlborough, MA). Mutagenesis was performed with the Transformer™ Mutagenesis Kit (CLONTECH, Palo Alto, CA)

according to the manufacturer's instructions. All mutations were verified by DNA sequencing using the Sequenase® V2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

<u>Table 1</u>

Primers:
Randomization of position 107:
CTC GGA GAC TCC C/GNN AAT CAT AAT AAA

Randomization of position 111:
GTA GTA GTA GTC C/GNN GGA GAC TCC CCG

Randomization of position 112:
GTC GTT GTA GTA GTA GTA GTA C/GNN CTC GGA GAC

<u>EXAMPLE 2</u>

<u>Preparation of extracts and BIAcore analysis of scFv Extracts:</u>

Mutagenized plasmids were introduced by electroporation into bacterial strain *Escherichia coli* TG1 for expression. Single colonies were inoculated into 10 ml of 2X-YT (which contains per liter of water 16 g tryptone, 10 g yeast extract and 5 g sodium chloride) supplemented with 2% glucose. Cells were grown overnight at 30°C with vigorous shaking, collected by centrifugation in a Beckman GPR centrifuge at 2500 rpm, and resuspended in 10 ml of fresh 2X-YT supplemented with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to induce expression. Cells were incubated at 30°C for an additional 5–6 hours with vigorous shaking, collected by centrifugation, resuspended in 1 ml of phosphate buffered saline: ethylenediametetraacetic acid (PBS:EDTA; 10 mM sodium phosphate pH7.0, 150 mM sodium chloride 1 mM EDTA), and incubated on ice for 30 minutes to

release periplasmic proteins. Extracts were clarified by centrifugation and stored at 4°C until use.

EXAMPLE 3

Off-rate determinations of the scFv antibodies were determined using the BIAcore system (Pharmacia Biosenser). HIV gp120 V3 loop peptides, Al-1 variant (Ala-1 peptide) were covalently immobilized on a carboxylated dextran/gold matrix via the primary amino group. The carboxyl-dextran matrix was first activated with N-ethyl-N'-(3-diethylaminopropyl)carbondiimide (EDC) and reacted with N-hydroxysuccinimide (NHS). HIV gp120 V3 loop peptides such as Ala-1 peptide were covalently immobilized via the free thiol of a cysteine placed at the N-terminus. These peptides were reacted with the EDC-NHS activated matrix which had been reacted with 2-(2-pyridinyldithio)ethaneamine. Remaining unreacted NHS-ester groups were displaced by addition of ethanolamine. EDTA extracts were added in a flow passing over the immobilized antigen. The refractive index changes, in the form of the surface plasmon resonance caused by the binding and subsequent dissociation of the scFv, were monitored continuously. Off-rates were calculated from the automatically collected data using the Pharmacis Kinetics Evaluation software.

EXAMPLE 4

Alanine scanning of CDR3 identifies residues which modulate scFv-antigen binding

Alanine scanning mutagenesis was used to identify residues within the VH CDR3 region of scFv clone P5Q critical for binding. It was hypothesized that effects on binding by alanine substitution would lead to four broad classes of effect: class i) slower off-rate; class ii) faster off-rate; class iii) loss of binding; and class iv) minor or no change in off-rate. Class i) and ii) were

operationally defined as critical. Class iii) was defined as obligatory. Class iv) was defined as noncritical.

The 27 positions that comprise VH CDR3 of scFv clone P5Q were individually changed to alanine by site-directed mutagenesis. Periplasmic extracts were prepared from the alanine replacement clones and assayed for off-rate determinations against the AL-1 gp120 V3 loop peptide (Fig. 1). Alanine substitutions at positions 107 and 111 resulted in 1.7 and 2.7 fold improvements in off-rate, respectively. These positions (class i) were judged critical and subsequently randomized to identify optimal residues. Alanine substitutions at positions 102, 112, 113, 114, and 118 led to faster off-rates (class ii); two of these positions were selected for further evaluation. Alanine substitution at positions 98, 101, 115, 116, 117, and 121 resulted in no binding (class iii). Alanine substitution at the remaining fourteen positions had only a minor effect on the off-rate (class iv). The class iii and iv positions were not evaluated further.

EXAMPLE 5

Randomization at critical positions to identify optimal amino acid solutions

The two critical class i) positions (107 and 111) were individually randomized to all amino acids, and off-rates against the AL-1 peptide determined. In addition, two class ii) positions (112 and 118) were also selected for randomization studies.

The results for position 107 are shown in Fig. 2. The slowest off-rate was observed with the negatively-charged glutamic acid, which decreased dissociation 2.5-fold. Substitution of other polar and charged amino acids had no significant effect on dissociation. With the exception of alanine, substitution with hydrophobic amino acid resulted in complete loss of binding. These results are consistent with the preponderance of surface ligand-contact residues being hydrophilic.

Randomization of position 111 (Fig. 3) showed that the aromatic residues tyrosine and tryptophan produced the slowest off-rates (dissociation rates decreased 4.2 and 4.7-fold, respectively). However, substitution with any hydrophobic amino acids increased affinity relative to wild-type clone P5Q.

Class ii) positions 112 and 118 (faster off-rate upon alanine substitution) were also selected for amino acid randomization. For both position 112 (Fig. 4) and 118, the residues present in the original scFv P5Q, aspartic acid and asparagine, were the best solutions.

EXAMPLE 6

Improvements at positions 107 and 111 are additive

A double mutant that combined the optimized residues at positions 107 (E) and 111 (W) was constructed to determine whether or not the individual improvements are additive. Figure 5 shows that the double mutant has an off-rate 9-fold slower than wild-type clone P5Q. The off-rate value approximates the product of the fold improvements observed with the individual optimized residues (2.5 for 107E and 4.7 for 111W). One interpretation of this result is that for these two positions, the contributions to scFv-antigen affinity are independent and additive.

EXAMPLE 7

Method of making modified antibodies

An antibody is mutagenized by alanine scanning mutagenesis to produce a modified antibody. The binding of the modified antibody to its antigen is determined. Binding determinations may be made by conventional methods and include off-rate measurements. Modified antibodies having desired characteristics are selected and maintained.

EXAMPLE 8

Method of using modified antibodies

The modified antibodies or pharmaceutical compositions thereof are used for the prophylactic or therapeutic treatment of diseases caused by their antigen. Methods of treatment include, but are not limited to, intravenous or intraperitoneal injection of the modified antibody.

EXAMPLE 9

Diagnostic kit employing modified antibodies

The modified antibodies of Example 7 are used as reagents in diagnostic kits. The modified antibody reagents may be further modified through techniques which are well-known in the art, such as radiolabeling or enzyme-labeling. The diagnostic kit may be used to detect or characterize the antigens.

EXAMPLE 10

DNA encoding modified antibodies

The DNA encoding the modified antibody of Example 7 is used as a reagent for the production of modified antibodies. The DNA may be incorporated into an expression vector. The expression vector may be used to transform a host cell. Cultivation of the host cell under conditions suitable for the expression results in the production of modified antibody.

EXAMPLE 11

DNA encoding modified antibodies

The DNA encoding the modified antibody of Example 7 is used to detect DNA encoding the antigen in test samples. Methods of detection include, but are not limited to, hybridization under selective conditions. Test samples include, but are not limited to, samples of blood, cells, and tissues.

EXAMPLE 12

Preparation of modified light chain immunoglobulins

The light chain of an immunoglobulin is mutagenized by alanine scanning mutagenesis to produce a modified immunoglobulin having modified binding characteristics. The modified immunoglobulin is used as a reagent for diagnostic kits or as a therapeutic agent.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LEWIS, CRAIG M.
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HOLLIS, GREGORY F.

(ii) TITLE OF INVENTION: IN VITRO ANTIBODY MATURATION

(iii) NUMBER OF SEQUENCES: 2

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(C) CITY: RAHWAY
(D) STATE: NJ
(E) COUNTRY: USA
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE: 04-MAR-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: CARTY, CHRISTINE E.
(B) REGISTRATION NUMBER: 36,090
(C) REFERENCE/DOCKET NUMBER: 19190P

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (908) 594-6734
(B) TELEFAX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 816 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTCAGACTCA CCTGTGTAGC CTCTGGCTTC ACGTTCAGTG ATGTCTGGCT GAACTGGGTC   120
CGCCAGGCCC CAGGGAAGGG GCTGGAGTGG GTCGGCCGTA TTAAAAGCGC CACTGATGGT   180
GGGACAACAG ACTACGCTGC ATCCGTGCAA GGCAGATTCA CCATCTCAAG AGATGACTCA   240
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AACGACGTTT GGGGCAAAGG GACCACGGTC ACCGTCTCCT CAGGTGCAGG CGGTTCAGGC   420
GGAGGTGGCT CTGGCGGTGG CGGATCGCAG TCTGTGTTGA CGCAGCCGCC CTCAGTGTCT   480
GCGGCCCCAG GACAGAAGGT CACCATCTCC TGCTCTGGAA GCAGCTCCAA CATTGGGAAT   540
AATTATGTAT TGTGGTACCA GCAGTTCCCA GGAACAGCCC CCAAACTCCT CATTTATGGC   600
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GCCACCCTGG GCATCACCGG ACTCCAGACT GGGGACGAGG CCGATTATTT CTGCGCAACA   720
TGGGATAGCG GCCTGAGTGC TGATTGGGTG TTCGGCGGAG GGACCAAGCT GACCGTCCTA   780
GGTGCGGCCG CAGAACAAAA ACTCATCTCA GAAGAG                             816
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 272 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ala Met Ala Glu Val Glx Leu Val Glu Ser Gly Gly Gly Leu Val Lys
1               5                   10                  15

Pro Gly Gly Ser Leu Arg Leu Thr Cys Val Ala Ser Gly Phe Thr Phe
            20                  25                  30

Ser Asp Val Trp Leu Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
        35                  40                  45

Glu Trp Val Gly Arg Ile Lys Ser Ala Thr Asp Gly Gly Thr Thr Asp
    50                  55                  60
```

Tyr Ala Ala Ser Val Gln Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
65 70 75 80

Lys Asn Thr Leu Tyr Leu Glx Met Asn Ser Leu Lys Thr Glu Asp Thr
85 90 95

Ala Val Tyr Ser Cys Asn Thr Asp Gly Phe Ile Met Ile Arg Gly Val
100 105 110

Ser Glu Asp Tyr Tyr Tyr Tyr Tyr Asn Asp Val Trp Gly Lys Gly Thr
115 120 125

Thr Val Thr Ala Ser Ser Gly Ala Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro Ser Val Ser Ala
145 150 155 160

Ala Pro Gly Gln Lys Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn
165 170 175

Ile Gly Asn Asn Tyr Val Leu Trp Tyr Gln Gln Phe Pro Gly Thr Ala
180 185 190

Pro Lys Leu Leu Ile Tyr Gly Asn Asn Lys Arg Pro Ser Gly Ile Pro
195 200 205

Asp Arg Phe Ser Gly Ser Lys Leu Leu Ile Tyr Gly Ala Thr Leu Gly
210 215 220

Ile Thr Gly Leu Gln Thr Gly Asp Gln Ala Asp Tyr Phe Cys Ala Thr
225 230 235 240

Trp Asp Ser Gly Leu Ser Ala Asp Trp Val Phe Gly Gly Gly Thr Lys
245 250 255

Leu Thr Val Leu Gly Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu
260 265 270

WHAT IS CLAIMED IS:

1. A DNA molecule encoding a modified antibody, the modified antibody being derived from a native antibody by alanine scanning mutagenesis and the modified antibody having binding characteristics different than binding characteristics of the native antibody.

2. The DNA molecule of Claim 1 wherein the native antibody is MAb447.

3. The DNA molecule of Claim 2, the DNA molecule being selected from the group consisting of P5Q, DNA encoding modified antibodies of Figures 1, 2, 3, 4, 5, combinations thereof, derivatives thereof and degenerate variants thereof.

4. A method of modifying an antibody to make an modified antibody comprising replacing at least one amino acid of the antibody with alanine to produce a modified antibody.

5. The method of Claim 4 wherein the modified antibody has improved binding characteristics.

6. Modified antibodies produced by the method of Claim 4 or homologues thereof.

7. The method of Claim 4 wherein the antibody is MAb447.

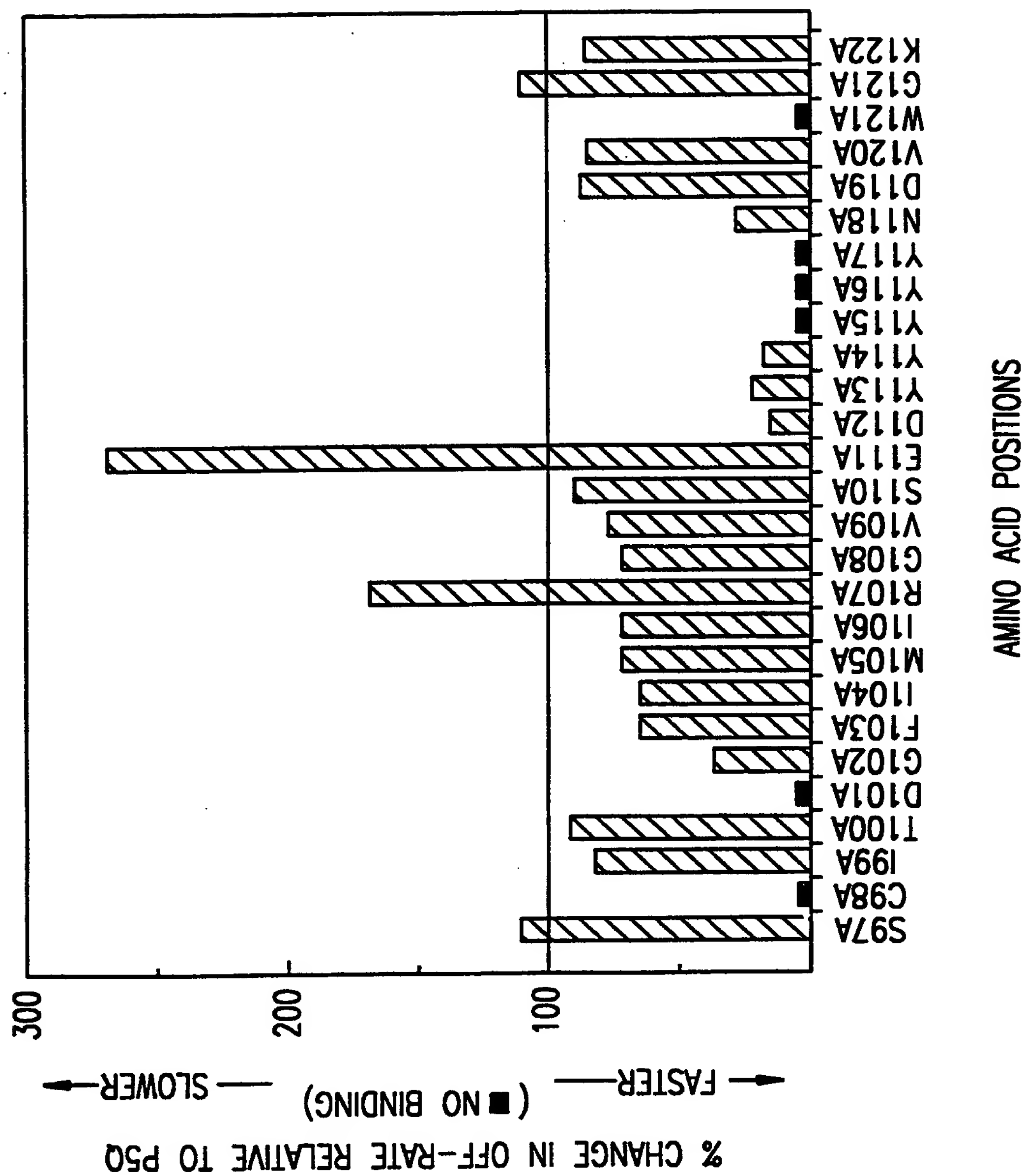
8. The method of Claim 7 wherein the amino acid replaced with alanine is located in complementary determining region 1, complementary determining region 2 or complementary determining region 3 of MAb447.

9. The modified antibodies of Claim 6 selected from the group consisting of P5Q, the antibodies of Figures 1, 2, 3, 4, 5, combinations thereof, derivatives thereof, and homologues thereof.

10. Diagnostic kits comprising the modified antibodies produced by the method of Claim 6.

11. Diagnostic kits comprising the DNA molecules of Claim 1.

12. A pharmaceutical composition comprising at least one modified antibody of Claim 6 or DNA encoding at least one modified antibody of Claim 6 or combinations thereof.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(■ NO BINDING)
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100
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C98A
I99A
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D101A
G102A
F103A
I104A
M105A
I106A
R107A
G108A
V109A
S110A
E111A
D112A
Y113A
Y114A
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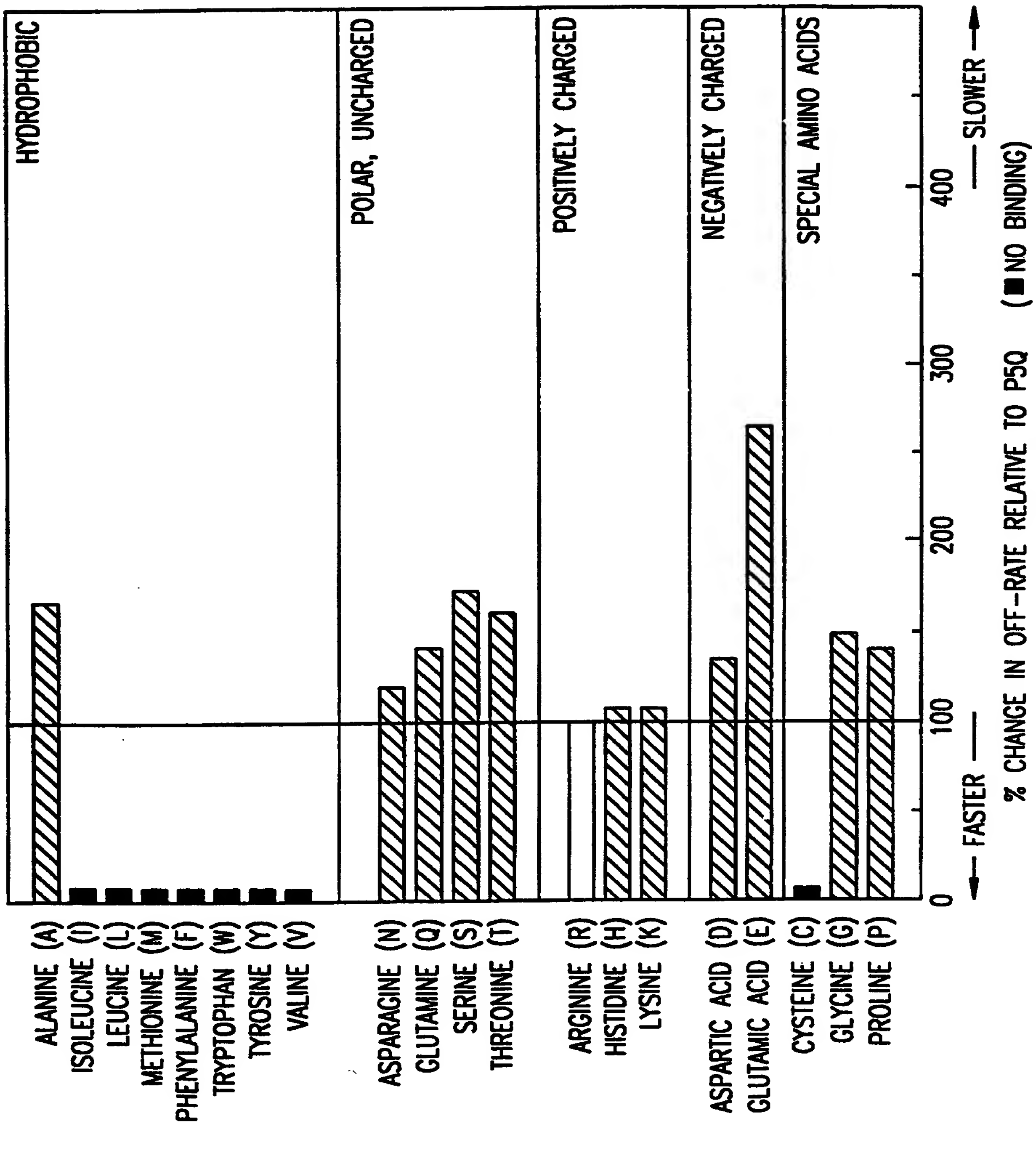
K122A
AMINO ACID POSITIONS

FIG.1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ISOLEUCINE (I)
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METHIONINE (M)
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ASPARAGINE (N)
GLUTAMINE (Q)
SERINE (S)
THREONINE (T)
ARGININE (R)
HISTIDINE (H)
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ASPARTIC ACID (D)
GLUTAMIC ACID (E)
CYSTEINE (C)
GLYCINE (G)
PROLINE (P)
HYDROPHOBIC
POLAR, UNCHARGED
POSITIVELY CHARGED
NEGATIVELY CHARGED
SPECIAL AMINO ACIDS
0
100
200
300
400
FASTER
SLOWER
% CHANGE IN OFF-RATE RELATIVE TO P5Q (■ NO BINDING)

FIG.2

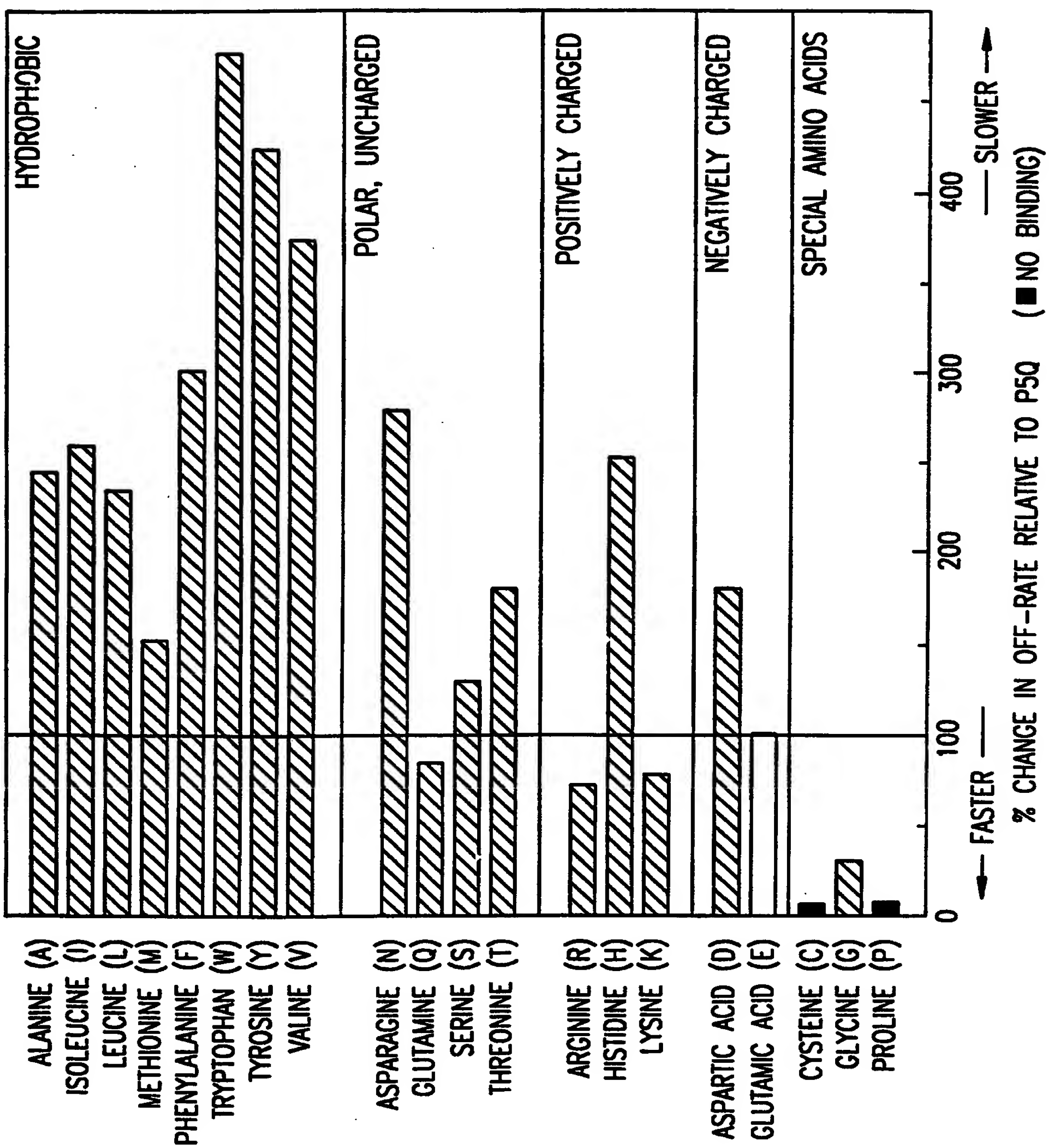
HYDROPHOBIC
POLAR, UNCHARGED
POSITIVELY CHARGED
NEGATIVELY CHARGED
SPECIAL AMINO ACIDS
ALANINE (A)
ISOLEUCINE (I)
LEUCINE (L)
METHIONINE (M)
PHENYLALANINE (F)
TRYPTOPHAN (W)
TYROSINE (Y)
VALINE (V)
ASPARAGINE (N)
GLUTAMINE (Q)
SERINE (S)
THREONINE (T)
ARGININE (R)
HISTIDINE (H)
LYSINE (K)
ASPARTIC ACID (D)
GLUTAMIC ACID (E)
CYSTEINE (C)
GLYCINE (G)
PROLINE (P)
0
100
200
300
400
← FASTER
SLOWER →
% CHANGE IN OFF-RATE RELATIVE TO P5Q (■ NO BINDING)

FIG.3

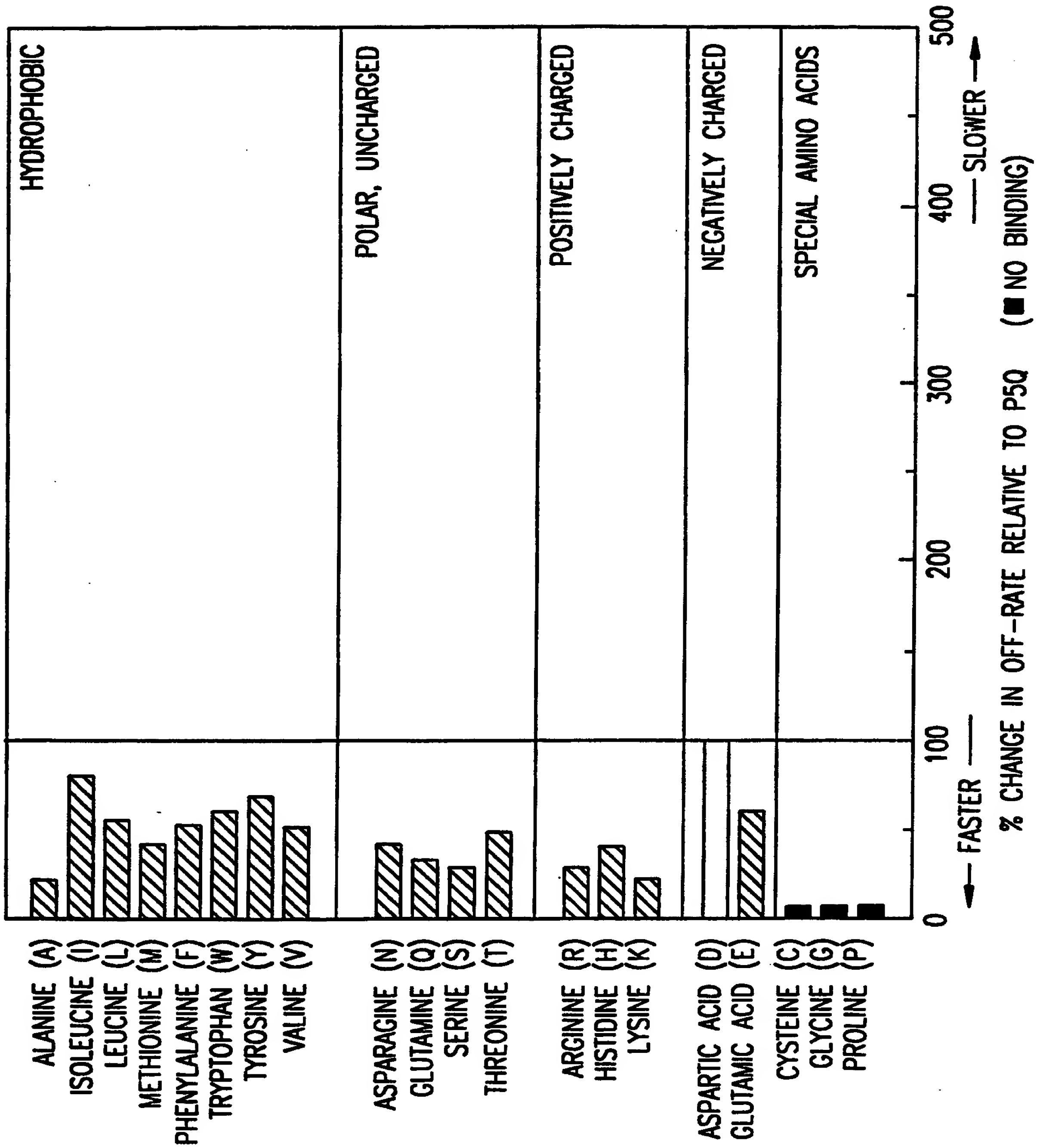
HYDROPHOBIC
POLAR, UNCHARGED
POSITIVELY CHARGED
NEGATIVELY CHARGED
SPECIAL AMINO ACIDS
ALANINE (A)
ISOLEUCINE (I)
LEUCINE (L)
METHIONINE (M)
PHENYLALANINE (F)
TRYPTOPHAN (W)
TYROSINE (Y)
VALINE (V)
ASPARAGINE (N)
GLUTAMINE (Q)
SERINE (S)
THREONINE (T)
ARGININE (R)
HISTIDINE (H)
LYSINE (K)
ASPARTIC ACID (D)
GLUTAMIC ACID (E)
CYSTEINE (C)
GLYCINE (G)
PROLINE (P)
0
100
200
300
400
500
FASTER
SLOWER
% CHANGE IN OFF-RATE RELATIVE TO P5Q (■ NO BINDING)

FIG.4

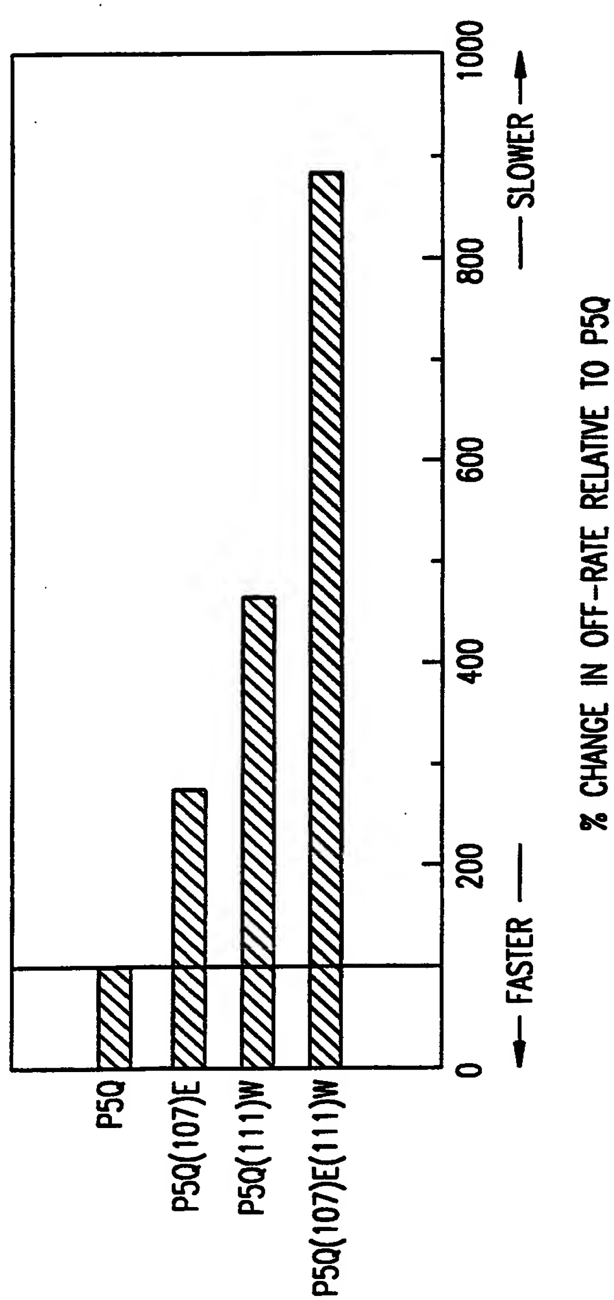
P5Q
P5Q(107)E
P5Q(111)W
P5Q(107)E(111)W
0
200
400
600
800
1000
← FASTER
SLOWER →
% CHANGE IN OFF-RATE RELATIVE TO P5Q

FIG.5

```
                 10                  20                  30                  40                  50                  60
                  *                   *                   *                   *                   *                   *
GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTA AAG CCT GGG GGG TCC
Ala M t Ala Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser

                 70                  80                  90                 100                 110                 120
                  *                   *                   *                   *                   *                   *
CTC AGA CTC ACC TGT GTA GCC TCT GGC TTC ACG TTC AGT GAT GTC TGG CTG AAC TGG GTC
Leu Arg Leu Thr Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Val Trp Leu Asn Trp Val

                130                 140                 150                 160                 170                 180
                  *                   *                   *                   *                   *                   *
CGC CAG GCC CCA GGG AAG GGG CTG GAG TGG GTC GGC CGT ATT AAA AGC GCC ACT GAT GGT
Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Gly Arg Ile Lys Ser Ala Thr Asp Gly

                190                 200                 210                 220                 230                 240
                  *                   *                   *                   *                   *                   *
GGG ACA ACA GAC TAC GCT GCA TCC GTG CAA GGC AGA TTC ACC ATC TCA AGA GAT GAC TCA
Gly Thr Thr Asp Tyr Ala Ala Ser Val Gln Gly Arg Phe Thr Ile Ser Arg Asp Asp S r
```

FIG.6a

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```
                250         260         270         280         290         300
                  *           *           *           *           *           *
AAA AAC ACG CTA TAT CTG CAA ATG AAT AGC CTG AAA ACC GAG GAC ACA GCC GTT TAT TCC
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr Ser

                310         320         330         .340        350         360
                  *           *           *           *           *           *
TGC AAC ACA GAT GGT TTT ATT ATG ATT CGG GGA GTC TCC GAG GAC TAC TAC TAC TAC TAC
Cys Asn Thr Asp Gly Phe Ile Met Ile Arg Gly Val Ser Glu Asp Tyr Tyr Tyr Tyr Tyr

                370         380         390         400         410         420
                  *           *           *           *           *           *
AAC GAC GTT TGG GGC AAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GCA GGC GGT TCA GGC
Asn Asp Val Trp Gly Lys Gly Thr Thr Val Thr Ala Ser Ser Gly Ala Gly Gly Ser Gly

                430         440         450         460         470         480
                  *           *           *           *           *           *
GGA GGT GGC TCT GGC GGT GGC GGA TCG CAG TCT GTG TTG ACG .CAG CCG CCC TCA GTG TCT
Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro Ser Val S r
```

FIG.6b

```
                490         500         510         520         530         540
                  *           *           *           *           *           *
GCG GCC CCA GGA CAG AAG GTC ACC ATC TCC TGC TCT GGA AGC AGC TCC AAC ATT GGG AAT
Ala Ala Pro Gly Gln Lys Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly Asn

                550         560         570         580         590         600
                  *           *           *           *           *           *
AAT TAT GTA TTG TGG TAC CAG CAG TTC CCA GGA ACA GCC CCC AAA CTC CTC ATT TAT GGC
Asn Tyr Val Leu Trp Tyr Gln Gln Phe Pro Gly Thr Ala Pro Lys Leu Leu Ile Tyr Gly

                610         620         630         640         650         660
                  *           *           *           *           *           *
AAT AAT AAG CGA CCC TCA GGG ATT CCT GAC CGA TTC TCT GGC TCC AAG TCT GGC ACG TCA
Asn Asn Lys Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr S r

                670         680         690         700         710         720
                  *           *           *           *           *           *
GCC ACC CTG GGC ATC ACC GGA CTC CAG ACT GGG GAC GAG GCC GAT TAT TTC TGC GCA ACA
Ala Thr Leu Gly Ile Thr Gly Leu Gln Thr Gly Asp Glu Ala Asp Tyr Phe Cys Ala Thr
```

FIG.6c

```
                 730         740         750         760         770         780
                   *           *           *           *           *           *
TGG GAT AGC GGC CTG AGT GCT GAT TGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA
Trp Asp Ser Gly Leu Ser Ala Asp Trp Val Phe Gly Gly Gly Thr Lys Leu Thr Val L u

                 790         800         810
                   *           *           *
GGT GCG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG
Gly Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu
```

FIG.6d

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US95/02492

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/00, 16/46; A61K 39/00; C12N 15/12, 15/13
US CL :424/133.1, 144.1; 536/23.53; 530/387.3
According to International Patent Classificati n (IPC) or to b th national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/133.1, 144.1; 536/23.53; 530/387.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SEQUENCE SEARCH, MEDLINE, EMBASE, LIFESCI, BIOSYS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
Y	J. IMMUNOLOGY, VOL. 150, NO. 2, ISSUED 15 JANUARY 1993, M.K. GORNY ET AL., "REPERTOIRE OF NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES SPECIFIC FOR THE V3 DOMAIN OF HIV-1 GP120", PAGES 635-643, SEE ENTIRE DOCUMENT.	1-12
Y	PROC. NATL. ACAD. SCI. USA, VOL. 87, ISSUED SEPTEMBER 1990, A. ASHKENAZI ET AL., "MAPPING OF THE CD4 BINDING SITE FOR HUMAN IMMUNODEFICIENCY VIRUS BY ALANINE-SCANNING MUTAGENESIS", PAGES 7150-7154, SEE ENTIRE DOCUMENT.	1-12
Y	SCIENCE, VOL. 244, ISSUED 02 JUNE 1989, B.C. CUNNINGHAM ET AL., "HIGH-RESOLUTION EPITOPE MAPPING OF hGH-RECEPTOR INTERACTIONS BY ALANINE-SCANNING MUTAGENESIS", PAGES 1081-1085, SEE ENTIRE DOCUMENT.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date f the actual completion f the international search	Date of mailing of the international search report
06 MAY 1995	23 MAY 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Auth rized officer CHRISTOPHER EISENSCHENK T lephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★